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PRINCIPAL INVESTIGATOR: Gerardi Evans, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

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Gerardi Evans, Ph.D.

E-Mail: gevan@cc.ucsf.edu

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Introduction

Identifying the optimal molecular targets for effective and specific treatment of breast carcinoma is limited by our ignorance of which molecular pathways or network nodes are critical for the initiation, evolution and, above all, maintenance of breast cancers. Our overarching hypothesis is that although tumors appear genetically complex, they most probably are dependent upon a very much more limited repertoire of mutations for their maintenance. To test this hypothesis, we proposed to construct a novel type of mouse cancer model in which endogenous genes encoding critical signaling molecules are modified so that there expression can be toggled on and off at will by the action of ligand-dependent heterologous repressors. Using such heterologous repressor targeting (HRT), we will directly ascertain the requirement for such signaling molecules in normal breast epithelial development and maintenance and for mutant forms of such molecules in driving and maintaining breast cancers. Our initial focus within the BCRP proposal is on c-*myc* and *e2f3* genes, both of which encode pleiotropic transcription factors whose deregulated activities are causally implicated in breast (and other) cancers.

Our proposal has three principal aims, each divided into a number of discrete tasks. The principal aims are:

1.   Construct genetically modified mice in which the endogenous c-*myc* and *e2f3* genes are rendered susceptible to ectopic control by either the IPTG-dependent Lac or tetracycline-dependent tTS^{Kid} repressors, so allowing their reversible repression (and subsequent re-expression) at any stage of breast tumor development. Engineering such mice includes several steps marked as "parallel," which represent "ideal" refinements to the model system that will be undertaken in parallel but are not be absolutely required for preliminary studies on the roles of c-*myc* and *E2f3* target genes in breast cancer.
2.   Use HRT-modified mice to determine directly the requirement for c-Myc and/or E2F3 at various stages of tumor development in an H-Ras-driven mouse model of breast cancer and evaluate the therapeutic utility of inhibiting c-Myc and/or E2F3 function in the treatment of breast cancer.
3.   Establish the HRT technology platform as a means to assess the requirement for c-Myc and/or E2F3 in any orthotopic mouse model of breast cancer.

Aim 1
Task a.   Construct LacI repressor c-Myc knock-in constructs and verify by sequencing (months 1-4).

This task has been completed, although unplanned and unforeseen complications somewhat delayed execution of this task.

Task b.   Electroporate constructs into ES cells (transgenic core). Develop screening protocols (southern and PCR) to identify correctly targeted ES cells (months 4-5).

This task has now been completed. Screening protocols were successfully developed and validated that allow for identification of correctly targeted ES cells. To facilitate this, a coding-neutral change in the 5′ sequence of c-*myc* exon 3 has been introduced into both targeting vectors that will allow for one-step

discrimination between targeted and endogenous c-*myc* (marked by the asterisk). Purified DNAs have been electroporated into ES cells and postive recombinant clones have been identified.

Task c.   Select and expand ES cells and identify ES cells with correctly targeted knock in element (months 5-7).
        This task has now been completed. ES cells with targeted c-*myc* have been identified and cloned. Appropriate recombination has been verified by the strategies outlined above.

Task d.   Verify IPTG-dependent repression of c-myc expression in ES cells in vitro using TaqMan analysis specific for the modified c-myc transcript (month 8).
        This task has largely been completed. Verification is underway and looks very encouraging. Endogenous c-*myc* expression has been tested and appears normal in the absence of IPTG administration.

Task e.   Microinject ES cells into blastocysts (UCSF transgenic core) and generate chimeric mice (months 9-11)
        This task has been completed. Targeted ES cells have been microinjected into blastocysts to generate appropriate knock-in chimeric mice.

Task f.   Breed positive chimeric mice to obtain germ line transmission of the modified c-myc gene (months 12-15).
        This task is underway.

Task g.   Start cross with Zp3Cre mice to excise Neo cassette left in the modified c-myc gene (month 14)
        To be completed.

Task h.   Cross LacI-c-Myc KI with beta-actin-(NLS)LacI (months 15-17)
        To be completed. The *beta-actin-(NLS)LacI* have been derived and are now available.

Task i.   Cross HRT LacI-c-cMyc X beta-actin-(NLS)LacI into WAP-H-Ras mice to generate the WAP-H-Ras breast cancer model with IPTG-switchable c-myc (HRT LacI-c-myc X Wap-H-Ras mice) (months 17-20)
        To be completed.

Task j.   Begin process of backcrossing of chimeras into C57/Black6 background (will need 6 generations, eventually)
        To be completed.

Task k.   Expand colony of HRT LacI-c-myc X Wap-H-Ras mice.
        To be completed.

Task 2

Construct mice in which the endogenous c-*myc* gene is rendered switchable by the Doxycycline-dependent-*tTS^{Kid}* repressor and establish this in the *WAP-H-Ras* mammary tumor background.

a.   Generate Tet repressor c-Myc knock in construct and verify by sequencing (months 1-4)
This task has been completed. Like the LacI vector, construction of the tTS^{Kid} HRT targeting vector required a number of complex cloning steps, including multiple three-way blunt ended ligations which, again, necessitated sorting through large numbers of products to identify correctly ligated vectors.

b.   Electroporate construct into ES cells (UCSF transgenic core). Develop screening protocols (southern and PCR) to identify correctly targeted ES cells (months 4-5).
Transfection of the new targeting vector into ES cells has been completed.

c.   Select and expand ES cells. Identify ES cells with correctly targeted knock in element (months 5-7).
Completed

d.   Verify doxycycline-dependent repression of c-myc in ES cells in vitro by Taqman expression analysis (month 8).
In progress.

e.   Microinjection ES cells into blastocysts (transgenic core) and production of chimeras (months 9-11).
Positive ES cell clones are currently being verified prior to micro-injection into blastocycts.

f.   Breed positive chimeric mice to obtain germ line transmission of the modified c-myc gene (months 12-15).
        To be completed.

g.   [parallel] Start cross with Zp3Cre mice to excise Neo cassette left in the modified c-myc gene (month 14).
        To be completed.

h.   Cross HRT-tet-c-cMyc X beta-actin-tTS^{Kid} mice (months 15-17).
        To be completed.

i.   Cross HRT-tet-c-cMyc X beta-actin-tTS^{Kid} into WAP-H-Ras mice to generate the WAP-H-Ras breast cancer model with Tet-switchable c-myc (HRT tTS^{Kid}c-myc X WAP-H-Ras mice) (months 17-20).
        To be completed.

j.   [parallel] Backcross of chimeras to C57Black6 (6 generations).
   To be completed.

k. *Expand colony of HRT tTS^{Kid}c-myc X WAP-H-Ras mice.*
  To be completed.

Task 3

Construct mice in which the endogenous *E2f3* gene is rendered switchable by the IPTG-dependent LacI repressor and establish this in the WAP-H-Ras mammary tumor background.

a. *Generate LacI repressor E2F3 knock in construct and verify by sequencing (months 1-4).*
  The *e2f3* vector required more complex cloning strategies than initially considered. This was due to to problems with obtaining the *e2f3* genomic sequence from the correct mouse strain but also because emerging evidence indicated that the *e2f3* gene encoded not one but two separate proteins, each with its own discrete set of properties. Most notably, *e2f3a* is a transcriptional activator that drives cells into cycle whereas the shorter *e2f3b* is a transcriptional repressor that damps down expression of the ARF tumor suppressor. In light of these unforeseen discoveries, the strategy was modified to generate vectors that would modify the endogenous *e2f3* gene to allow independent regulation of *e2f3a* and *e2f3b*. The extensively modified targeting vector is depicted in Figure XX. As is evident from this diagram, we have inserted a Tet-regulatable sequence just upstream of the *e2f3a* transcriptional start site that should, in principle, shut down expression of both *e2f3a* and *e2f3b* transcripts. In addition, *loxP* sites have been inserted either side of *e2f3a* exon 1 while *flt* sites frame exon 1 of *e2f3b*. Thus, we will be able selectively to permanently ablate expression of either *e2f3a* or *e2f3b*, and then

Figure 1
Diagrammatic representation of *e2f3* targeting vectors
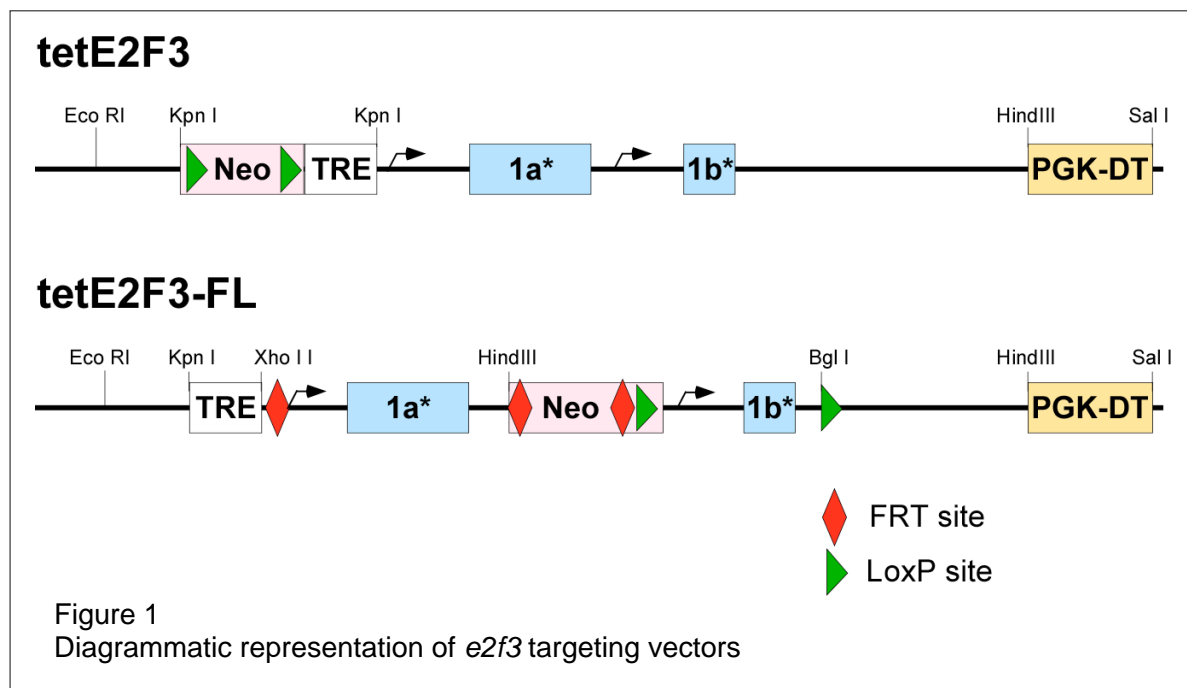
superimpose on this real-time regulation of the remaining e2f3 transcript.

b.   Electroporate construct into ES cells (UCSF transgenic core). Develop screening protocols (southern and PCR) to identify correctly targeted ES cells (months 4-5).
With successful completion of the targeting vector, transfections into ES cells are currently underway.

c.   Select and expand ES cells and identify ES cells with correctly targeted knock in element (month 5-7).
Execution of this task is pending isolation of transfected ES cells.

d.   Validate IPTG-dependent repression of E2F3 in ES cells in vitro by Taqman expression analysis (month 8).
Execution of this task is pending isolation of transfected ES cells.

e.   Microinject ES cells into blastocysts (transgenic core) and production of chimeras (months 9-11).
Execution of this task is pending isolation of transfected ES cells.

f.   Breed positive chimeric mice to obtain germ line transmission of the modified E2f3 gene (months 12-15).
To be completed.

g.   [parallel] Start cross with Zp3Cre mice to excise Neo cassette left in the modified E2f3 gene (month 14).
To be completed.

h.   Cross LacI-E2f3 KI with beta-actin-(NLS)LacI (months 15-17).
To be completed.

i.   Cross HRT LacI-E2f3 X beta-actin-(NLS)LacI into WAP-H-Ras mice to generate the WAP-H-Ras breast cancer model with IPTG-switchable E2f3 (HRT LacI-E2f3 X Wap-H-Ras mice) (months 17-20).
To be completed.

j.   [parallel] Begin process of backcrossing of chimeras into C57Black6 background (will need 6 generations, eventually).
To be completed.

k.   Expand colony of HRT LacI-E2f3 X Wap-H-Ras mice.
To be completed.

Task 4
Determine effect of c-Myc and E2F3-inactivation on normal mouse tissues and on development and maintenance of H-*Ras*-induced breast adenocarcinoma *in vivo*

a.   Determine the systemic effects of temporary c-Myc ablation on mouse viability and susceptible tissues (skin, GI tract, bone marrow) after administering IPTG or Dox to, respectively, HRT LacI-c-myc and HRT tTS^{Kid}c-myc mice for varying lengths of time (2 days-2 weeks) (months 20-36).
      To be completed.

b.   Determine the systemic effects of temporary E2F3 ablation on mouse viability and susceptible tissues (skin, GI tract, bone marrow) after administering IPTG to HRT LacI-E2f3 mice for varying lengths of time (2 days-2 weeks) (months 20-36).
      To be completed.

c.   Determine effects of short term (as defined in a. and b. above as the longest time that c-Myc or E2F3 can be repressed without significant pathology) on palpable H-Ras-induced breast tumors. Histological and immunohistochemical analysis of tissue samples from adenocarcinomas (months 20-36).
      To be completed.

KEY RESEARCH ACCOMPLISHMENTS:

•   Successful design, construction and targeting of vector to render endogenous c-*myc* gene regulatable via the IPTG-dependent Lac Repressor. Construction of targeted mouse is proceeding ahead of schedule.

•   Successful design, construction and targeting of vector to render endogenous c-*myc* gene regulatable via the tTS^{kid} tetracycline-regulatable. Construction of targeted mouse is on schedule.

•   Successful construction and checking of modified targeting vector. Modifications required because of new discoveries concerning the nature of gene products encoded by the *e2f3* gene and their disparate biological functions.

•   Successful establishment of initial colony of *beta-actin-(NLS)LacI* mice on schedule. Animals now inside quarantine barrier at UCSF.

REPORTABLE OUTCOMES:

None as yet.

CONCLUSIONS:

We have successfully implemented much of groundwork needed to test our

biological hypotheses. Currently, progress is dependent upon difficult to control factors like mouse breeding. However, direct analysi of the roles of c-*myc* and *e2f3* function in the genesis, progression and maintenance of H-Ras-induced breast cancer in mice should be achieved within the next months.

REFERENCES:
None